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Rapid Detection of o-Hydroxycinnamic Acid and Beta-Glucosidase in *Melilotus alba*

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Rapid Detection of *o*-Hydroxycinnamic Acid and Beta-Glucosidase in *Melilotus alba*[1]

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ABSTRACT

The tests utilize small samples of leaf tissue crushed on sheets of filter paper. For detection of *o*-hydroxycinnamic acid (*o*-HCA), a crude preparation containing sweetclover β-glucosidase is added to a spot of crushed tissue to insure hydrolysis of glucosidically bound *o*-HCA. An extract containing *cis-o*-HCA glucoside is added to another tissue spot to serve as the substrate for the detection of β-glucosidase activity. Finally, all spots are moistened with NaOH and are scored for fluorescence under ultraviolet light. Results of qualitative and quantitative tests on plants representing various genotypes are compared. The importance of exposing plants to sunlight before testing for *o*-HCA is discussed. The tests are particularly useful in genetic studies, in the development and maintenance of breeding lines, and in testing for contamination in low-*o*-HCA sweetclover varieties.

Additional index words: **Sweetclover, Coumarin.**

SEVERAL qualitative and quantitative fluorometric procedures for the assay of coumarin and related compounds in sweetclover (*Melilotus alba* Desr.) are discussed in the review of Smith and Gorz (14). Of the qualitative procedures cited in that review, the most recent were published in 1958 (2, 12). Biosynthetic studies published since 1958 have contributed greatly to an improved understanding of such details of the coumarin pathway as the relationship of the glucosides of *cis-* and *trans-o*-hydroxycinnamic acid (*o*-HCA) to free and bound coumarin, the role of the enzyme, β-glucosidase, in the pathway, and the control of *o*-HCA level and β-glucosidase activity, respectively, by the *Cu/cu* and *B/b* allelic pairs (5). This understanding, in turn, has permitted the development of improved qualitative tests for both *o*-HCA and β-glucosidase. In this report, the modified tests currently in use in this laboratory are described, and results of their application are presented and discussed.

Sweetclover plants of the *CuCu* genotype are relatively high in content of the glucosides of *cis-* and *trans-o*-HCA; *Cucu* plants are intermediate; and *cucu* plants are low in content (3, 11). Similarly, preparations of *BB* plants are high in β-glucosidase activity, those of *Bb* plants are intermediate, and *bb* preparations appear to be inactive (6). Experience has shown that the qualitative tests for *o*-HCA content and β-glucosidase activity are highly reliable in cases involving plants homozygous for the *Cu/cu* and *B/b* genes, but that some difficulty in classification may be expected if the plant population includes individuals heterozygous for either or both of the two genes. For this reason, plants of all possible genotypes with respect to these two allelic pairs were included in this study.

MATERIALS AND METHODS

Plant Material. Lines of biennial, white-blossomed sweetclover (*M. alba*) of the *CuCuBB, CuCubb, cucuBB,* and *cucubb* genotypes were used. The derivation of these four closely related lines has been described (3). Seed of all heterozygous genotypes except *Cucubb* was obtained from appropriate crosses among the homozygous lines; thus, with the exception noted, the heterozygous individuals were very closely related to the homozygous

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lines. The *Cucubb* seed resulted from a cross in which the male parent (*CuCubb* genotype) was somewhat less closely related to the other plants.

Seeds (one per carton) were planted in 1-pint, plastic-coated milk cartons containing a mixture of vermiculite, soil, and sand (5:5:1). The resulting plants were grown in growth chambers under continuous cool white fluorescent light (approximately 10,000 lux), at approximately 27C and 50% relative humidity. Plants were pruned as necessary to prevent flowering and to promote branching so that the supply of young leaves would be adequate for the assays.

One day prior to sampling, plants were taken from the chambers and were exposed to sunlight under a partly cloudy sky. This exposure was necessary to convert *trans-o*-HCA glucoside, present in chamber-grown plants, to the *cis* form (8). Sweetclover β-glucosidase, which was used in the qualitative test for *o*-HCA, is specific for the *cis* isomer (10, 13).

Qualitative Tests. Sheets of Whatman No. 1[3] filter paper, measuring 15 by 25 cm, were used. Lines were printed on the sheets, dividing them into 2.5-cm squares. Plants to be sampled were arranged in an order such that successive samples came from different genotypes. This sort of situation might be encountered in sampling a segregating population, for example. A young leaf, still slightly folded and not yet fully grown, was selected from each plant. With a knife blade or small spatula, a portion of leaf tissue approximately 5 to 10 mm^2 in area was crushed at the center of each of two adjacent squares on the filter paper sheet. Test papers were placed on sheets of waxed paper for this crushing, and were kept there for subsequent treatment. Immediately after each test sheet was filled with samples, one of the sample spots from each plant was moistened with a crude β-glucosidase preparation (enzyme) and the other with a crude extract containing *cis-o*-HCA glucoside (substrate). Fine-tipped pipettes were used to apply the enzyme and substrate solutions. Following application of these solutions, test sheets were air-dried. All sample spots were then moistened with 2.5*N* NaOH, applied with a fine-tipped pipette; then the sheets were exposed immediately to ultraviolet light from the T.F.8 tube (energy peak near 360 nm) of a Gates M.R.4 Laboratory Lamp. Fluorescence scores were assigned as follows: H, intense yellowish-green fluorescence throughout the area of the sample spot; H—, distinctly fluorescent but with less intensity than H samples, darker areas usually present toward outer edges of sample spot; L+, slight fluorescence, more yellow than the typical *o*-HCA fluorescence; L, virtually no fluorescence. Scoring was done while samples were still moist from the addition of NaOH solution. Interfering fluorescence intensified if sheets were allowed to dry completely prior to scoring.

The enzyme preparation was obtained from young leaves of field-grown *cucuBB* plants. The fresh leaf tissue was ground in a mortar with water (2 ml/g of leaves) and a small amount of sea sand. The ground material was centrifuged at 35,000 × *g* for 20 min at approximately 3 C, and the supernatant fraction was decanted and saved. The pellet was resuspended in cold water (2 ml/g original leaves) and recentrifuged. The combined supernatant fractions were filtered through muslin to remove suspended particles. One-ml portions of the resulting preparation were pipetted into individual 12 × 75-mm test tubes for freezing. As needed, these tubes were thawed, and 3 ml of water was added to each to provide a satisfactory concentration of enzyme for treating the sample spots. Assay of β-glucosidase activity indicated that 1 ml of the undiluted preparation had the capacity to hydrolyze 6.6 μmoles of *cis-o*-HCA glucoside per minute.

The substrate solution was derived from young leaves of *M. officinalis* (L.) Lam. 'Goldtop.' Young leaves of any sweetclover plants of the *Cu* phenotype would be satisfactory for this purpose. Fresh leaves were immersed in boiling water (5 ml/g of leaves) and autoclaved for 20 min at 120 C. The extract was cooled, filtered, and freeze-dried. Assay of the dried powder indicated contents of 85 μg of *cis-o*-HCA and 10 μg of *trans-o*-HCA per mg, present as the respective glucosides. This powder was dissolved in water to provide a substrate solution containing the equivalent of 525 μg of *cis-o*-HCA per ml.

To check for possible contamination of enzyme and substrate solutions, a separate check spot of each was applied to each of the test sheets. Also, enzyme solution was applied to a dried spot of substrate on each sheet. Fluorescence was expected only from those check spots to which both enzyme and substrate were applied.

Quantitative Assays. Samples for quantitative determination of *o*-HCA content and β-glucosidase activity were harvested within a few minutes after the qualitative test samples had been taken from the respective plants.

For *o*-HCA assay, the youngest fully expanded leaf was taken from each branch which had served as a source of leaf tissue for the qualitative tests. The sampled leaves were extracted with hot water and assayed for *trans* and total *o*-HCA as previously described (7).

The β-glucosidase assay sample for each plant consisted of the next to the youngest fully expanded leaf from the same branch which had supplied the samples for qualitative tests and *o*-HCA determination, plus four young leaves (as near as possible to the youngest fully expanded stage) from other branches on the same plant. Each of these 5-leaf samples was weighed and each was ground in a mortar with a small amount of sea sand. After the tissue was thoroughly pulverized, buffer (0.05*M* Tris-HCl, pH 8.5) was added at the rate of 1 ml/100 mg of fresh tissue, and grinding was continued for 1 or 2 min. Slurries were centrifuged for 5 min in a clinical centrifuge. The crude supernatant fractions were held in a freezer until assays could be accomplished. The procedure used in assaying these crude preparations for β-glucosidase activity was identical to that described earlier (6), except that the substrate concentration was approximately 40% higher in the present assays. Preparations of *bb* leaves were assayed without further dilution; those of *Bb* and *BB* leaves were diluted 40-fold with the Tris-HCl buffer prior to assay.

RESULTS AND DISCUSSION

A summary of the results of the qualitative and quantitative assays is presented in Table 1. As in previous experiments conducted here (3) and at other laboratories (11), *cucu*, *Cucu*, and *CuCu* plants were low, intermediate, and high, respectively, in content of *o*-HCA. The range in *o*-HCA content in *cucu* plants did not overlap the range in *Cucu* or *CuCu* plants, but there was a small amount of overlap between the *Cucu* and *CuCu* ranges. The data given represent total *o*-HCA levels; that is the sum of the *cis* and *trans* isomers. On the average, approximately 17% of the total *o*-HCA in these samples was present as the *trans* isomer.

The qualitative scores for *o*-HCA content were based on the fluorescence of the spots of crushed leaf tissue to which sweetclover β-glucosidase was applied. Application of this enzyme was required to hydrolyze *cis-o*-HCA glucoside, and was particularly important in permitting the correct scoring of *Cu-bb* plants. Without the added enzyme, the qualitative test would not distinguish *Cu-bb* plants from *cucuB-* or *cucubb* individuals.

As shown in Table 1, *cucu* plants were clearly distinguished from the *CuCu* and *Cucu* genotypes by the qualitative test; in fact, none of the *cucu* plants appeared high enough in *o*-HCA content to merit

Table 1. Comparison of quantitative assays and qualitative scores of *o*-hydroxycinnamic acid and β-glucosidase activity in plants representing nine genotypes.

		o-Hydroxycinnamic acid					β-glucosidase activity				
	No. of	Quantitative*		Qualitative score			Quantitative†		Qualitative score		
Genotype	plants	Mean	Range	L	H-	H	Mean	Range	L	L+	H- H
cucubb	11	0.16	0.09-0.21	11			< 0.01	0.00-0.01	11		
cucuBB	11	0.17	0.07-0.22	11			6.56	5.76-7.46			11
CuCubb	11	5.41	3.23-8.86			11	< 0.01	0.00-0.01	7	3	1
CuCuBB	11	5.52	4.22-6.77		1	10	6.17	4.60-7.76			11
cucuBb	13	0.17	0.10-0.26	13			3.24	2.56-3.79		4	9
Cucubb	10	2.74	1.79-3.18		9	1	0.01	0.01-0.02	8	2	
CucuBB	13	2.23	1.75-3.00		13		6.31	5.72-6.83			13
CuCuBb	11	5.98	4.85-7.22		1	10	2.94	1.93-3.68			11
CucuBb	19	1.94	1.23-3.49		15	4	3.04	2.49-3.97		1	18

* % of dry weight. † Micromoles of substrate hydrolyzed per min per ml enzyme preparation.

[3] Mention of specific products is for identification only and does not imply endorsement of the product by USDA.

assignment above the L category. The *CuCu* and *Cucu* plants were assigned predominantly to the H and H— classes, respectively, but the distinction between genotypes was not completely clear. This result is not surprising in view of the overlap observed in the quantitative *o*-HCA assays. Instances of overlap or near overlap were not restricted to the *Cucu* plants classified as H or the *CuCu* plants scored as H—.

Attempts were made to use the commercially available β-glucosidase, almond emulsin, in the qualitative test, but these met with only limited success. The sweetclover enzyme preparation gave clearer results, probably because this enzyme is highly specific for *cis-o*-HCA glucoside (10, 13), the isomer which predominates in field- and greenhouse-grown plants (1, 9). Almond emulsin, on the other hand, hydrolyzes the *trans* isomer more readily than the *cis* (10, 13). At most stations where a qualitative test for *o*-HCA is needed in sweetclover screening or breeding work, plants of the *cuB* phenotype probably would be available for use in making a sweetclover β-glucosidase preparation. *CuB* plants also could be used if the β-glucosidase were separated from the endogenous substrate by precipitation of the enzyme with acetone or $(NH_4)_2SO_4$ (10, 13).

Quantitative assays for β-glucosidase activity (Table 1) agreed with published results (6) in that preparations of *bb*, *Bb*, and *BB* plants were very low, intermediate, and high, respectively, in activity. Plants of the *bb* genotype were readily distinguished from *B*— plants, but the most active preparations from *Bb* plants were almost as active as the least active ones from *BB* plants.

The qualitative test for β-glucosidase was based on the spots to which extract containing *cis-o*-HCA glucoside was applied. Application of this substrate permitted the detection of β-glucosidase activity whether or not endogenous substrate was present; that is, regardless of the constitution of the plants with respect to the *Cu/cu* alleles. The distinction between *bb* and *B*— plants was less clear than that between *cucu* and *Cu*— plants. Thus, of the 32 *bb* plants tested, five were given scores of L+ and one was scored H— in the qualitative test, despite the fact that very little if any β-glucosidase activity was detected in any of these plants by the quantitative assay. The L+ scores were not particularly disturbing, but the H— score was an obvious misclassification for a *bb* plant. The reason for the high score on this plant is not understood. The qualitative test was not reliable for distinguishing between *BB* and *Bb* plants. All except five of the 43 *Bb* plants tested were scored as H and, thus, were not distinguished from the 35 *BB* plants, all of which were classified as H.

A study conducted in conjunction with the investigation of *Cu* dominance (3) furnished additional information on the reliability of the qualitative test. All of the plants assayed quantitatively in that investigation also were tested qualitatively for *o*-HCA. The tests, made first on a sunny day, were repeated on a cloudy day. The test distinguished between *cucu* and *CuCu* plants with a high degree of accuracy under cloudy as well as sunny conditions (Table 2). However, the distinction between *cucu* and *Cucu* plants was acceptable only when testing was done under sunny conditions. The stereospecificity of sweetclover

Table 2. Qualitative *o*-hydroxycinnamic acid scores of *cucu*, *Cucu*, and *CuCu* plants under sunny and cloudy conditions.

Genotype	No. of plants	Condition	Qualitative o-HCA score			
			L	L+	H-	H
cucu	197	Sunny	196	0	1	0
		Cloudy	180	15	2	0
Cucu	198	Sunny	3	2	135	58
		Cloudy	10	75	103	10
CuCu	199	Sunny	1	0	12	186
		Cloudy	1	3	66	129

β-glucosidase and the influence of sunlight on *cis-trans* isomerization of *o*-HCA glucoside are at least partially responsible for the observed dependence of test results upon light conditions. Testing should not be done during, or immediately following, periods of darkness or heavy cloudiness.

These rapid, simultaneous tests for *o*-HCA and β-glucosidase activity have been useful in several ways. For example, in the recent study on contamination in the low-*o*-HCA variety, Denta, these methods were used to good advantage in testing several thousand plants for both *o*-HCA and β-glucosidase (4). The test for *o*-HCA has been used in the development and maintenance of low-*o*-HCA breeding lines. Both tests have excellent potential for use in genetic experiments in which the *Cu/cu* and *B/b* alleles are employed as markers. Finally, the tests are highly useful in checking the phenotype of plants which are to be used for biochemical studies.

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